THE VETERINARY JOURNAL

Editors :

T. DALLING, M.A., M.R.C.V.S.
J. McCUNN, M.R.C.V.S., L.R.C.P.

MARCH, 1946.

EDITORIAL

ANIMAL HEALTH

THE subject of animal health was referred to by the Minister of Agriculture and Fisheries in the House of Commons during a recent debate. He spoke of animal husbandry—livestock improvement—as "the very keystone of our agricultural economy": he said that "the threefold improvement policy is a consistent campaign to get farmers to use good stock and sound breeding, eradication of disease, and up-to-date stock management and economical feeding, which means making the best use of such feeding stuffs as are available": he referred to "the unremitting fight which has been going on for some time to reduce crippling losses which have affected farmers for so long," and to the shortage of veterinary surgeons as "a handicap which has been slowly removed as veterinary surgeons come back from the armed Forces," but added, "many more are needed."

It has long been recognised that in this country we have some of the best livestock obtainable, and that their influence on improving animals in other parts of the world is sought: the recent large prices paid for bulls shows clearly how our cattle are valued. It behoves all concerned, therefore, to ensure that everything possible is done to maintain the quality of livestock throughout the country. Sound breeding policies are essential, and the use of good quality sires is of fundamental importance. While we have many breeders of national and international reputation in this country, there is room for improvement in much of the stock of the average farmer. As artificial insemination becomes more popular, and facilities for its use more general, farmers will be able to have a breeding policy in their herds which should lead to considerable improvement. There is

XUM

little doubt that artificial insemination practices will prove of real value to the agricultural community. In addition to an improvement in the quality of cattle following its adoption, it will also go some considerable distance in controlling some of the important diseases transmissible *by coitus*. Animal husbandry, management and feeding whereby economical use of feeding stuffs is practised will all play considerable parts in the condition and quality of our livestock: steps are being taken whereby the farming world will receive guidance on these and other subjects.

In addition, however, to all these important aspects of the livestock industry, the health of the animal must be considered of paramount importance: without a healthy livestock population and the adoption of sound methods to maintain health and to control disease, many of the other practices in livestock improvement are bound to fall short. Animal health is a vast subject and one that calls for study by men and women who have been trained in different branches of science. We, veterinarians, have a great responsibility to bear, for it is to us that the agricultural world looks eventually for guidance and assistance in the matter of animal health. Research work on fundamental processes is essential in order that we can appreciate the "healthy" tissues and systems: in this work there is room for much development and for close collaboration between scientists of different schools and training. The study of the abnormal or "diseased" tissue, system or process must also claim the attention of those trained in many aspects of science: for some of these important studies, however, training in veterinary science is very necessary. The application of the results of research and study of methods for the control of health and disease must fall to the Veterinary Profession, whose daily work is among the various types of livestock. It may be felt by some that our knowledge of methods for the control of animal health and disease is far short of requirements: it is certainly true that many problems still face us, but, on the other hand, it can be rightly claimed that to-day our knowledge of disease and its control is enabling us to practise methods which are yielding results of much economic value. The study of the various health and disease problems is no light task: as more and more encouragement is given to this work, and as more and more scientists with their different training and experiences become involved in the work, so surely will our knowledge improve and new, and better, methods of control be devised. Research work must be a conjoint effort of all who can assist in shedding light on the various problems which confront the livestock industry.

XUM

General Articles

PS. PYOCYANEA: STUDY *IN VITRO* AND *IN VIVO* OF THE BACTERICIDAL AND THERAPEUTIC PROPERTIES OF α-OXYPHENAZINE AND A LIPOIDAL PRODUCT

By J. ZWEIG, *Ministry of Agriculture and Fisheries, Veterinary Laboratory, Weybridge.*

Pseudomonas pyocyanea (*Ps. p.*) occupies a prominent place in the early literature of bacterial antagonism. The first serious attempt to employ an antibiotic for curative purposes was that of Emmerich and Loew (1899), who demonstrated the antagonistic properties of *Ps. p.* against *B. anthracis in vitro* and *in vivo*. These antibacterial properties were confirmed *in vitro* and *in vivo* by Woodhead, M. M., and Wood, C. (1889), and Vaerst, K. (1902). Bouchard, C. (1889), also reported good results from the inoculation of *Ps. p.* into guinea-pigs and rabbits simultaneously with or shortly after inoculations of *B. anthracis*. Morphological changes produced in *B. anthracis* were described by Emmerich, R., and Saida (1900). Emmerich, Loew and Korschum (1902) drew attention to the fact that old cultures of *Ps. p.* were highly bactericidal to many micro-organisms, and noticed that such old cultures concentrated ten times *in vacuo* had the interesting property of causing quick and complete lysis of *V. choleræ* and *B. anthracis*. This action was ascribed to an enzyme which they termed "Pyocyanase." The enzymic nature of the antibiotic, suggested by Emmerich as early as 1899, was contradicted by other workers (Klimoff, 1901, Dietrich, 1901). "Pyocyanase," 3-4 week old *Ps. p.* cultures killed by chloroform or toluene, filtered, and finally reduced to 1/10 of their volume *in vacuo*, was prepared on a large scale in the Saxon Serum Institute, in Dresden, and was on sale in Germany till as late as 1936. It was mainly utilised therapeutically in combating infections which are insusceptible to local treatment, e.g., infections of the mucous membranes (angina, diphtheria) and certain diseases of the urino-genital system and skin. Opinion regarding the value of this commercial preparation varied, Hettche (1931-32) attributing its disinfectant property to the phenol added as a preservative. Sterilised cultures of *Ps. p.*, 15-30 days old, were used parenterally with some success in anthrax infections in guinea-pigs by Fortineau (1910) and by the same author (1914) in human beings.

The first attempt to isolate the active part of *Ps. p.* cultures was made by Raubitschek and Russ (1908), the "lipoids" obtained by alcoholic extraction exhibiting considerable bactericidal action on *B. anthracis*. Fukuhara (1909) and Ohkubo (1910) confirmed the bactericidal action of alcoholic extracts and also showed that acetonic extracts were active. Hosoya (1929) experimented with a "lipoid" in crystalline form. Grundel and Wagner (1930, 1931) attributed the actions of the lipoids to fatty acids. Hettche (1933, 1934) isolated a liquid acid, split, in his opinion, from neutral fats by a lipase produced in *Ps. p.* cultures, and called it "pyocyanic acid." Birch and Hirschfeld (1935) found the alcohol, ether and chloroform, but not the petrol-ether extract, active. Stokes, Peck and Woodward (1942) claimed good results *in vitro* with an ether soluble fatty material and showed that it had little or no correlation with the gram staining abilities of the test organism. In addition to the "lipoid," another bactericidal metabolite is

XUM

present in older *Ps. p.* cultures, viz.: α-oxy-phenazine. This is a derivative of pyocyanine, and was isolated and later synthesised by Wrede and Strack (1924, 1928, 1929). Schoental (1941) isolated three antibacterial substances from chloroform extracts of *Ps. p.* cultures: (1) Pyocyanine, (2) α-oxy-phenazine, and (3) an almost colourless bactericidal lipoidal substance, and compared their antibacterial activities *in vitro* with those of other disinfectants. Stokes, Peck and Woodward (1942) compared the bactericidal potency of α-oxy-phenazine with pyocyanine and so-called pyocyanase.

OWN INVESTIGATIONS

The present work was undertaken to study methods of preparation and the chemical and antibiotic properties *in vitro* and *in vivo* of various metabolites obtained from *Ps. p.* cultures, with special attention to α-oxy-phenazine and the lipoidal products against organisms of veterinary importance.

Methods of Preparation

Several strains of *Ps. p.* were investigated in the early stages of this study, but the highly pyocyanogenic strain O was selected for all subsequent work. This strain was smooth and its colonies very viscous. A pronounced bacteriophage-like phenomenon showed itself on solid media by central lysis and corroded margins of the colonies, the lysed areas having a metallic sheen.

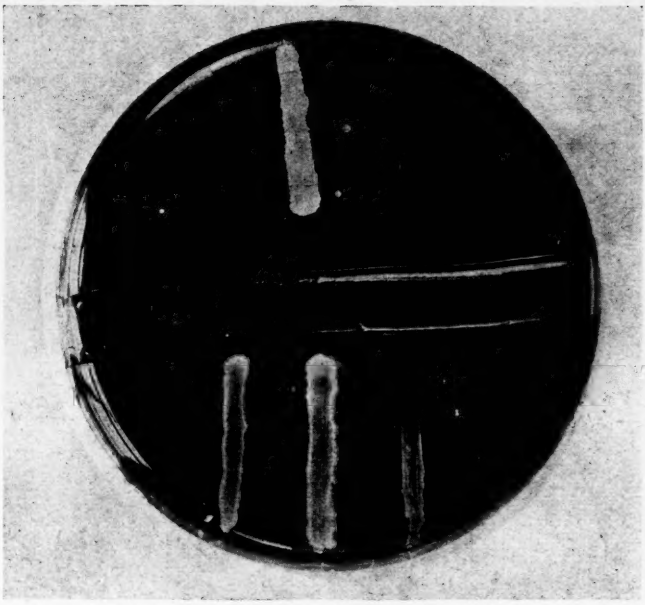
α-oxy-phenazine.

α-oxy-phenazine can be obtained direct from old cultures, but it was found more economical to prepare it by the conversion of the pyocyanine produced in young cultures. *Ps. p.* strain O was seeded into Roux flasks, each containing 140 c.c. of 2 per cent. glycerine-agar (Lemco meat extract 0.5 per cent.) at pH. 7.5. The cultures were incubated for 4-7 days. The pyocyanine was extracted by repeated washings with chloroform and then, by means of separating funnels, taken into acidified water. This watery solution of pyocyanine chloride was stable and large quantities for later transformation into α-oxy-phenazine were accumulated. The demethylation of pyocyanine was accomplished by strong alkalinisation and standing for 48 hours, or usually gently heating until a red-violet colour appeared. The solution (cooled, if it had been warmed) was then acidified with acetic acid and 3-4 times extracted with ether, dehydrated with anhydrous sodium sulphate and filtered through cotton wool. The ether was distilled off, the residue taken into benzene-absolute alcohol åå, filtered and the solvent evaporated. The residue was then taken into benzene, filtered, evaporated and finally dissolved in small amounts of hot 70 per cent. alcohol. This alcoholic solution was cooled and filtered. Crystallisation occurred after careful addition of water and constant stirring. The yellow, crystalline α-oxy-phenazine was collected in Buchner's funnels and 2-3 times purified by recrystallisation.

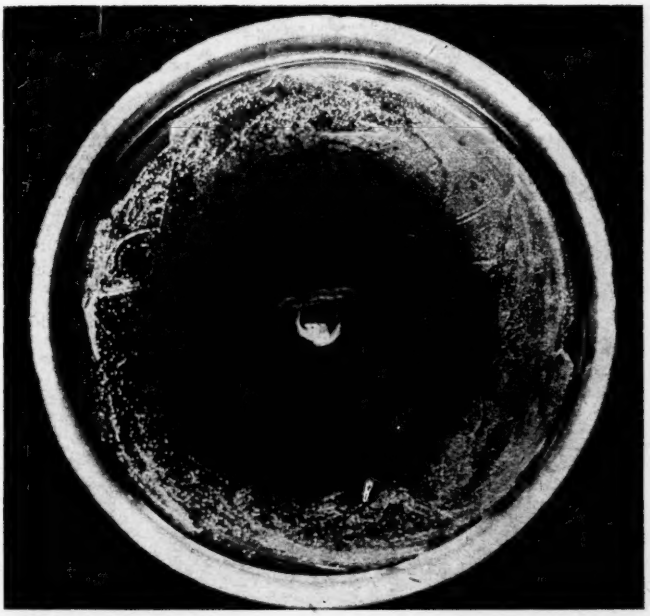
Lipoidal Products.

In the early stages of the work dried bacterial bodies of *Ps. p.* grown on solid media were used for extraction by chloroform in a Soxhlet apparatus. Later it was found that the use of dried whole broth cultures was much more economical, the active material obtained being qualitatively identical with that obtained by the previous method.

2 per cent. glycerine beef infusion (without peptone), containing 0.5 per cent. sodium chloride at pH. 7.5, was used and the cultures incubated for 1-2 months. The pellicle was broken up by shaking every second day to allow autolysis of sunken bacterial bodies. Extreme difficulty was met in extracting the cultures



α-oxyphenazine " ditch plate."
Left to right, upper row: Str. dysgalactiæ, Staph. aureus (Oxford), Str. agalactiæ.
Left to right, lower row: S. choleræ suis, E. coli, Past, septica (bovine).



Culture of Pseudomonas pyocyanca in centre of plate simultaneously spread with *Br. abortus*—544 (aërobic strain).

XUM

owing to their viscous nature, but by lowering the p.H. with phosphoric acid to 3.5 this was overcome. 400 gr. of ammonium sulphate was added to each litre and after filtration the reddish liquid was 3 times extracted by chloroform, the unwanted pyocyanine-chloride being left in the water phase. A litre yielded by this method 300-400 mg. of crude lipoid. At a later stage it became possible to use a freeze-drying plant and 1-2 months' old Ps. p. cultures were acidified and then dried by this method. The dried material was collected and extracted in the Soxhlet apparatus, 600-650 mg. of crude lipoid being obtained from each 200 c.c. of original culture. The crude lipoid, obtained by either of the two methods described, was dried by distilling off the chloroform, and purified by resolution in alkaline water and conversion by basic lead acetate into its ether-soluble lead salt. Both products were yellow in colour, the purified one being much lighter and of an oily, semi-solid nature, characterised by a strong pineapple-like smell. There was no demonstrable loss of activity when it was kept in a vacuum desiccator over calcium-chloride or under nitrogen for 2 months. (No assay was carried out covering a longer period).

The purified lipoid was much more active in comparative tests *in vitro*, but the crude form was used for all *in vivo* experiments because of the large amounts needed and its much simpler production.

In Vitro Assay

Technique.

Except in the case of *Br. abortus*, the test organism was grown in liquid medium (meat medium for the anærobes, serum broth for other organisms), centrifuged at the time of optimal growth and the sediment washed and re-suspended in a volume of normal saline equal to that of the original culture. *Br. abortus* was grown on glycerine-dextrose agar, suspended in normal saline and adjusted to tube No. 3 of Brown scale. The bacterial suspensions were diluted to give about 1,000 viable organisms in the final test inoculum. The product under test was dissolved in a alkalinised serum-broth, neutralised and the bactericidal action titrated, as follows: a row of falling dilutions of the antibacterial agent was set up in serum broth (5 per cent.) at pH. 7.5 to give, after the addition of 0.2 c.c. of the test organism suspension, final concentrations ranging from 1 in 100 to 1 in a million. These mixtures were kept at 37° C. and samples of 0.2 c.c. were explanted after 15 minutes, 30 minutes, 1, 2, 3, 4 and 5 hours into tubes containing 4 c.c. of serum—or, in some cases, blood-agar (both 5 per cent.)—kept melted at 48° C. in a water bath. The inoculated tube was then rolled under running cold water until the agar had solidified as a thin film on the wall of the tube. Counts were made after the required incubation, when necessary, in a 10 per cent. CO_2 atmosphere or oxygen-free conditions in McIntosh and Fildes jars. The results for α-oxy-phenazine are summarised in Table I, for the lipoidal product in Table II, and for the reconstituted, heat killed, and freeze-dried whole *Ps. p.* culture in Table III.

IN VIVO EXPERIMENTS

Br. Abortus Infections.

The sensitivity of *Br. abortus* to both α-oxy-phenazine and the lipoid product in *in vitro* experiments and their high therapeutic ratio (toxic dose: minimal effective dose) made it desirable to make *in vivo* studies in experimental *Br. abortus* infections in guinea-pigs, and, as far as was possible, in natural bovine infections.

XUM

TABLE I.

Dilutions of α-oxy-phenazine at which bactericidal and bacteriostatic effects were observed. Inhibition zones on ditch plates. (Fleming, 1929.)

Bacterial species	Time and dilutions (mg/ml) at which bactericidal effects were observed.							Bacteriostatic action during	Inhibition zone on ditch plate	Remarks
	15 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	5 hrs.		
C. pyogenes	1.0	1.0	0.1	0.002	0.002	0.002	0.002	0.001	14 mm	
Vibrio fœtus	0.05	0.05	0.02	0.01	0.01	0.01	0.01	0.001	12 „	
Pasteurella bovis	1.0	1.0	1.0	0.002	0.002	0.002	0.002	0.001	7 „	
B. anthracis	1.0	1.0	0.2	0.1	0.1	0.005	0.02	0.002	12 „	
Str. agalactiæ	1.0	1.0	0.2	0.1	0.02	0.02	0.02	0.01	12 „	Partly bacteriostatic at 0.002
Str. dysgalactiæ	1.0	1.0	0.2	0.1	0.1	0.02	0.02	0.01	10 „	
Staph. aureus	2.0	2.0	1.0	1.0	1.0	0.2	0.02	0.01	9 „	
Br. abortus	0.2	0.1	0.05	0.05	0.05	0.05	0.05	0.02	10 „	
E. coli	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.02	1.5 „	
S. choleræsuis	1.0	1.0	0.2	0.2	0.2	0.2	0.2	0.1	1.0 „	
Cl. welchii	1.0	0.5	0.5	0.5	0.2	0.2	0.2	0.1	6 „	
Cl. septique	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.2	6 „	Partly bacteriostatic at 0.1
Cl. chauvoei	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2	6 „	
Cl. tetani	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.2	6 „	

Note of strains used: C. pyogenes E-850, V. fœtus-864, Past. bovis-723, B. anthracis-34 F2, Str. agalactiæ 119, Str. dysgalactiæ-52, Staph. aureus Oxford, Br. abortus-544 (CO_2 sensitive), Erysipelathrix rhusiopathiæ-Elmslie, E. Coli-231, Sal. choleræ suis Lytle, Cl. Welchii-Solo., Staph. aureus (mastitis)-421.

TABLE II.

Dilutions of the lipoidal product at which bactericidal and bacteriostatic effects were observed, and inhibition zones on ditch plate.

Bacterial species	Time and dilutions of Lipoid (mg/ml) at which bactericidal effects were observed.							Bacteriostatic action during	Inhibition zone on ditch plate	
	15 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	5 hrs.		
B. anthracis	1.0	0.5	0.05	0.02	0.01	0.01	0.01	0.002	12 mm	
Br. abortus	1.0	0.5	0.1	0.1	0.1	0.05	0.05	0.02	7 „	
Str. agalactiæ	2.0	1.0	1.0	1.0	1.0	0.2	0.1	0.05	4 „	
„ „	1.0	1.0	1.0	0.2	0.2	0.2	0.2	0.05	6 „	(purified Lipoid)
Staph. aureus	2.0	2.0	1.0	1.0	1.0	0.2	0.2	0.2	4 „	
„ „	1.0	1.0	1.0	0.2	0.2	0.2	0.1	0.05	6 „	(purified Lipoid)
Staph. (bovine)	2.0	1.0	1.0	1.0	1.0	0.2	0.1	0.1	4 „	

TABLE III.

Dilutions of the reconstituted freeze-dried culture, at which bactericidal and bacteriostatic effects were observed. Inhibition zones on ditch plate.

Bacterial species	Time and dilutions per cent. at which bactericidal effects were observed.							Bacteriostatic action during	Inhibition zone on ditch plate	
	15 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	5 hrs.		
Str. agalactiæ	100	50	50	50	50	50	20	10	5 mm	partly bacteriostatic at 2 per cent.
Staph. aureus	100	100	50	50	50	20	20	10	5 „	

Experiments in Guinea-pigs

Experiment 1.

This experiment was carried out to determine (a) the relative activity of the complex antibiotic metabolites of *Ps. p.*, and (b) the most opportune period of the pathogenesis at which to intervene.

Products used: A. 2-months-old broth cultures, killed by chloroform, filtered (Seitz EK) and evaporated under reduced pressure to 1/10 volume.

B. Ditto, but heat killed (55° C. for 1 hr.) and not concentrated.

C. 14-days-old glycerine broth cultures, heat killed, containing mainly pyocyanine and toxins.

D. α-oxy-phenazine obtained by chloroform extraction of glycerine agar cultures. A watery solution obtained from the last ether phase (see method of preparation) was used, its concentration being estimated colorimetrically.

E. Crude lipoid: extracted from dried bacterial bodies by chloroform in the Soxhlet apparatus.

All products were adjusted to about pH. 7.5.

Tolerance Tests were first carried out by various routes on 62 mice and 16 guinea pigs, and showed that the amounts to be used for this experiment could be safely administered.

Technique. The guinea-pigs were infected by the intramuscular route in the right thigh and the biological product injected intramuscularly in the left thigh in order to avoid any direct local effect.

The guinea-pigs were divided into 5 main groups (A-E), one for each product as listed above. Each main group was sub-divided in to 3 sub-groups: the first sub-group was treated one hour before infection, the second one week, and the third two weeks after infection. The guinea-pigs were kept throughout the experiments in cages, each containing 1 or 2 guinea-pigs.

These sub-groups were further divided into two halves, each containing 5 guinea-pigs, the product concerned being injected once and twice (interval 2 days) respectively in the two halves of the sub-group.

Eight guinea-pigs were used as controls.

Test dose. 500 organisms of *Br. abortus* S.544, CO_2 sensitive, controlled by viability counts in Roll tubes and Photoabsorptiometer.

Dosage of therapeutic agent. For A, 1 c.c.; B, 2 c.c.; C, 1 c.c.; D and E, concentrations and quantities as present in B.

Autopsy. All guinea-pigs were killed 4 weeks after infection. The spleens and sacral-lumbar lymph glands were ground in Griffith tubes. 0.2 c.c. of spleen pulp dilutions of 1/3, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and lymph gland pulp diluted 1/3 were planted into liver agar Roll tubes. After 5 days' incubation in a 10 per cent. CO_2 atmosphere bacterial counts of *Br. abortus* were made.

Each guinea-pig was bled and the serum titrated by agglutination, standard *Br. abortus* agglutinating suspension being used (Stableforth, 1936). The results agreed in general with those obtained by culture, and are therefore omitted in the table, though some reference is made of them in the text.

The results are given in Table No. IV.

The routine technique was the same in all following *Br. abortus* investigations in guinea-pigs.*

* The test doses and methods of examination adopted were those in current use at Weybridge for tests of the vaccinating power of strains of *Br. abortus* (Stableforth, unpublished).

XUM

TABLE IV.

Summary of infection rate.

		Time of treatment after infection			Total of all 3 periods	Infection rate of both doses per product	
		0 days	7 days	14 days			
Combined	1 dose	3/3	3/3	2/3	8/9	15/20	75%
A Product (inspissated)	2 doses	2/3	1/3	4/5	7/11		
Combined	1 dose	1/2	4/4	4/4	9/10	16/21	76%
B Product (not inspissated)	2 doses	3/3	1/4	3/4	7/11		
Combined							
C Product	1 dose	4/4	2/5	3/4	9/13	23/29	79%
(14 days old—containing bact. bodies)	2 doses	7/7	3/4	4/5	14/16		
-oxy-	1 dose	2/5	2/5	3/5	7/15	16/29	55%
D phenazine	2 doses	2/4	2/5	5/5	9/14		
Bacterial	1 dose	2/5	4/5	3/4	9/14	16/29	55%
E Extract	2 doses	1/5	1/5	5/5	7/15		
Total of periods of all groups		27/41	23/43	36/44	One dose	42/61	69%
					Two doses	44/67	66%
D + E only		7/19	9/20	16/19	One dose	16/29	55%
					Two doses	16/29	55%
Controls		Untreated			8/8	100%	

Note: c.f. 1/5 means 1 guinea pig out of 5 remained infected (4 being bacteriologically sterile).

Comment on results. The test infection which was expected to give 90-100 per cent. infection actually infected all control guinea-pigs. There is a clear indication that all products exerted some curative effect. This was small in the case of products A, B and C, but marked in the case of α-oxy-phenazine and the bacterial extract, both having given similar results, viz., 55 per cent. infection rate, as against 100 per cent. in the controls. It also appears that the therapeutic intervention is best made during the first 7 days. There is also an indication in the bacterial extract that 2 doses at an interval of 2 days may be better than a single dose. In confirmation of the positive and negative results shown in Table IV there was in addition a striking difference in the counts obtained in the guinea-pigs which remained positive, viz., in groups D and E an average count of 132,000 per gram spleen, as compared with 480,000 per gram of controls.

The guinea-pigs showed marked depression for 2-3 days in the groups, A, B, and the dose of C had to be halved because of its high toxicity.

There were no signs of toxicity after the injections of D and E.

Experiment 2.

The information obtained in the previous experiment suggested the investigation of higher doses of E and D (100 mg. of bacterial extract per dose and 10 mg. of α-oxy-phenazine), injected the 7th day after infection.

Further groups of guinea-pigs, 10 each, were also included, in which the injection of bacterial extract (100 mg.) and α-oxy-phenazine (10 mg.), respectively, were repeated three times at intervals of 5 days.

All guinea-pigs were infected with 500 *Br. abortus,* as in Experiment 1.

Results. In the α-oxy-phenazine group, 2 out of 10 guinea-pigs remained infected (2 slightly positive in lymph glands, spleen sterile).

XUM

In the group in which α-oxy-phenazine was repeated all guinea-pigs died within two hours after the third injection. The α-oxy-phenazine injected at this occasion was afterwards found to have an alkalinity of nearly 10, and the deaths should be attributed to this cause. This error was found to be due to a defect in the pH. meter used at the time.

In the group treated once with 100 mg. of bacterial extract one guinea-pig only out of 7 was bacteriologically positive (100 organisms per gram of spleen and blood titre 1/10). In the sub-group treated 3 times with 100 mg. of bacterial extract 4 (1 slightly positive in lymph glands) out of 10 remained infected (average counts of organisms per ml. of spleen of the 4 guinea-pigs not cured were 9,794, against 816,920 and 100 per cent. infection rate in controls).

Experiment 3.

The purpose of this experiment was to determine :

(a) The relative value of 3 different doses of α-oxy-phenazine, viz., 50 mg. 25 mg. and 10 mg.

(b) The relative value of injections by the intramuscular and intraperitoneal route.

(c) The relative value of one or more injections. (Successive days in the case of the intramuscular route and alternate days in the case of the intraperitoneal route).

(d). To compare the cultural crystalline product with a synthetic product prepared elsewhere.*

The test dose was 5,000 organisms of *Br. abortus*, i.e., ten times that used in Experiments 1 and 2.

Results of the infection rate are given in Table V.

* *Note* : The synthetic product was prepared and supplied by Glaxo Laboratories, Ltd., through the kind offices of Dr. F. A. Robinson.

TABLE V.

Summary of Infection Rate.

Dose.	Route.	Number of Injections.	Infection Rate. Cultural Product.	Synthetic Product.
50 mg. ...	i/m.	3x	2/8	3/6
50 mg. ...	„	1x	3/6	3/6
25 mg. ...	„	3x	3/6	—
25 mg. ...	„	1x	6/8	—
10 mg. ...	„	3x	8/8	6/6
10 mg. ...	„	1x	8/8	6/6
50 mg. ...	i/p.	2x	3/3	4/5
50 mg. ...	„	1x	3/3	4/4
25 mg. ...	„	2x	8/8	—
25 mg. ...	„	1x	8/8	—
10 mg. ...	„	2x	8/8	5/5
10 mg. ...	„	1x	7/8	6/6
Controls : 20/20.				

XUM

Comment on results. (1) It is clear from Table V that no curative effect was obtained by the intraperitoneal route.

(2) The 10 mg. dose of α-oxy-phenazine was insufficient even by the intramuscular route.

(3) The results obtained with the 50 mg. dose injection once or three times and the 25 mg. dose injected three times were similar and a little over 50 per cent., that is, somewhat better than in Experiment 1, although the infective dose of 5,000 organisms of *Br. abortus* was 10 times as large.

(4) The cultural crystalline product and the synthetic product were of about the same value.

The spleen counts and titres of guinea-pigs which remained infected in the group to which reference is made under (3) and (4) above, were markedly lower than in the controls or the other treated guinea-pigs.

Experiments on Br. abortus Infections in Cows

Technique. Bacteriological and serological examination of milk samples was carried out daily for one month before and one month or more after treatment. During the first week after treatment samples from each quarter were taken daily a.m. and p.m. Gravity cream from 20 ml. milk samples, kept over night in the refrigerator, was spread in 0.03 ml. amounts by means of a standard pipette on to dye plates (malachite green 1/500,000, Gentian violet 1/250,000 in liver infusion agar), incubated for 7 days in 10 per cent. CO_2 and the bacterial count determined.

Whey was obtained by the coagulation of the milk by rennet, the sample being placed for 1 hour in the incubator. Standard *Br. abortus* suspension was used for testing.

Comment. Fourteen quarters excreting continuously large numbers of *Br. abortus* were treated by single infusions of lipoidal extracts or α-oxy-phenazine, in some cases assisted by intravenous injections. Although the bacterial counts fell considerably or became even completely negative up to 17 days, all eventually became positive, but in only two cases did they reach their pre-injection level.

Mastitis Experiments in Cows

Technique. Daily milk samples from each quarter were taken at least 1 month previous to experiment and at least 1 month following treatment. These samples were examined macroscopically and culturally by planting 0.1 ml. of the fresh sample on 5 per cent. blood agar plates. After 2 days' incubation of the plates the following nomenclature was adopted for the numbers of colonies found on each plate: "Some" (10-20), "many" (20-100), "numerous" (100 or more), and "innumerable." In addition, the samples were incubated over-night and one loopful plated on Edward's selective medium (crystal violet 1:500,000, æsculin 1:1,000, ox blood 5 per cent., in ordinary Lemco-agar pH. 7.5). Where plates from the original sample were negative, but streptococci were obtained from incubated samples, only the numbers were recorded as "few." In all cases the streptococcus in question was identified by its cultural and, where necessary, its biochemical characters, and also serologically by precipitation tests with specific group streptococcus sera.*

* Prepared at Weybridge by Mr. J. C. Buxton.

XUM

TABLE No. VI.

EXPERIMENTS IN COWS AFFECTED WITH *BR. ABORTUS*.

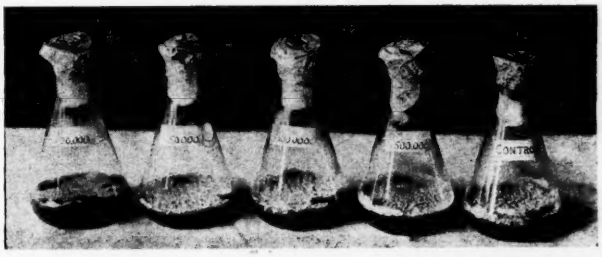
	History, whey titre, and bacterial count before treatment.					After Treatment.		
Cow.	Blood titre.	General history.	Whey titre.	Bacterial Count.	Treatment.	Blood titre.	Whey titre.	Bacterial Count.	Clinical Effect of Treatment. (Local and general.)
50′	1 :320	Aborted.	LF 1 :40	LF = 380 cols.	LF. 500 cc. *Combined Product* dilution 1 :5 in dist. water, left for 2 hrs.	1 :320	Rose to 1 :320 for 8 days, then gradual fall to 1 :80.	Few colonies for 2 days, neg. for 1 week then to pre-injection level.	Udder somewhat tense and sensitive at 2 hrs. In 18 hrs. much improved, few flocculi, 36 hrs. normal.
50[illegible]	1 :640	Aborted.	LF 1 :160 LH 1 :20 RF 1 :160 RH 1 :20	LF about RF 400 cols.	RF. 500 cc. *bacterial extract* 1 :1000 in dist. water, left for 1 hr.	1 :640	Rose to 1 :640 for 7 days, then gradual fall to 1 :80.	Sterile for 17 days, then positive again.	None whatever.
504	1 :320	Aborted.	LF 1 :80 LH 1 :200 RF 1 :40 RH 1 :60	LF 350 cols. LH 200 „ RF 450 „ RH 200 „	All quarters given *α-oxyphenazine* 500 cc., 1 :1000 left for 19 hrs. 1,000 cc. also injected intravenously in Ringer (= 1 gm.)	1 :320	(next day) LF 1 :1000 LH 1 :400 RF 1 :300 RH 1 :350 for 7 weeks.*	LF : 0,0,200,0,0,0, then erratically positive and negative. LH : 0,0,0, then to pre-injection level. RF : 100,0,0, then erratically positive. RH : 0,80,0,0, then erratically positive.	None whatever.
508	1 :640	Aborted.	LF 1 :320 LH 1 :20 RF 1 :320 RH 1 :20	LF 450 cols. RF	All quarters given 500 ml. *α-oxyphenazine*, 1 :1000 left for 18 hrs., 2 gm. given intravenously in Ringer's at 1 :500 conc.	1 :1280	(next day) LF 1 :1280 LH 1 :100 RF 1 :1280 RH 1 :160 for 3 month.*	0,0,100,200,0,0,0, then erratically positive and negative again.	None whatever.
457	1 :2560	Aborted.	LF 1 :1600 LH 1 :3200 RF 1 :1280 RH 1 :2500	Negative 5000 cols. 2000 „ 5000 „	All quarters given 500 ml. *Bact. Extract* 250 ml. of Bact. Extract (1 :500 in Ringer's sol.) intravenously.	1 :5120	(next day) LF 1 :4000 LH 1 :4000 RF 1 :4000 RH 1 :4400 for 5 weeks.*	LF neg. LH 500 for 8 days, then 2800, then persistent at 800 level. RF fall to 1500,500,300, 100,50, reaching 0 on the 15th day and thereafter. RH fall to 1000 col. level.	Little subcutaneous œdema, which disappeared in 24 hrs. Negligible change in secretion. No systemic disturbance.

Note.—Col. 5. Bacterial counts are per 0.03 ml. gravity cream.
* Col. 8. Tests not carried beyond 7 weeks, 3 months and 5 weeks respectively.
Col. 9. Counts are for successive days unless otherwise stated.

TABLE No VII.
EXPERIMENTAL COWS AFFECTED WITH BOVINE MASTITIS.

Cow.	Quarter.	Bacteriological diagnosis. description of milk, bacterial count before treatment. Bact. diagnosis.	Description of milk.	Bact. Count.	Treatment.	Bacteriological Count, description of milk after treatment. Bacterial Count.	Description of milk.	Local re-action from treatment
605	L.F.	Str. dysgalactiæ.	Little serous fluid, mass of gelatinous fleshy bloodstained pus.	Numerous to innumerable.	500 ml. of α-oxyphenazine (1 :10,000) in dist. water, left for 18 hrs.	Some, few (1 col) few, few, some neg. neg. neg. neg. few. few.	Second day: watery slightly opaque, few white floculi. Remained somewhat watery, but otherwise milk like after one week.	None
606	R.H.	Str. agalactiæ	Very little, thin, cloudy fluid, mass of heavy gelatinous whitish pus.	Many.	500 ml. of α-oxyphenazine (1 :10,000) in dist. water, left for 18 hrs.	few, few (1 col) few, many, few, few, few, some, few, neg. few and continued infected but well below pre-infection level.	second day: improved but numerous minute floculi, after one week few tiny floculi but otherwise normal milk.	None
608	R.H	Mixed infection Str. dysgalactiæ and Str. agalactiæ.	Little thin, blood-stained fluid, mass of gelatinous, yellowish markedly blood-stained pus.	For both organisms: Numerous to innumerable.	500 ml. of α-oxyphenazine (1 :1000) in dist. water, left for 18 hrs.	for both organisms: few, few, few, few, few (5 col.) few (3 col) few, few 16th free of Str. agal; Str. dysga'actiæ remained consistently at few.	second day: almost normal milk, very few minute floculi, became completely normal in 4 days.	None
607	L.H.	Str. agalactiæ.	Yellow pus.	Innumerable.	500 ml. of α-oxyphenazine (1 :500) in dist. water in all 4 quarters, left until calving.	continuous: numerous to innumerable Bacteriologically negative.	Normal colostrum and normal milk in 5 quarters, negligible improvement in L.H.	None
	R.H.	Str. agalactiæ.	Yellowish, thick, gelatinous mass.	Innumerable.				
307	L.F.	Str. agalactiæ.	Few, minute, white floculi.	Many.	500 ml. of α-oxyphenazine (1 :10,000) in 50% M/5 borate buffer.	(after one week) negative	Normal milk.	None and no drop in milk yield.
	L.H.			Many.		many		
	R.F.			Innumerable.		many		
	R.H.			Manv.		negative.		
					Reinfused in 3 weeks with α-oxyphenazine 1 :1000 in 50% M/5 borate buffer.	(next day) all rising few negative, later to some negative. pre-injection level	Normal milk.	None and no drop in milk yield.
606	R.F.	Str. agalactiæ.	Pale, cloudy, buff fluid, largish whitish clots.	Numerous to innumerable.	500 ml. of Bacterial Extract (1 :500) in dist. water. Left 1 hr.	few, few, few, some few, few numerous later continuous few, often only 1 col.	second day: watery fluid, some clots, and continued so.	None

Note.—607 treated when dry, L.F. and R.F. were bact. negative before treatment.
L.H. quarter was reinjected in 1 week without any change in the condition.
307 Synth. cryst. α-oxyphenazine was used.
Col. 7. The terms "few," "some," etc., refer to the results of daily examinations unless otherwise stated.



Serial dilutions of Ps. p. lipoid in synthetic medium seeded with Mycobacterium tuberculosis (human strain)—5 weeks' incubation.

Transplant of upper cultures after 7 weeks' incubation. Incubation time of viability test 4 weeks.

Col. 7. The terms "few," "some," etc., refer to the results of daily examinations unless otherwise stated.

XUM

Comment. From Table VII it will be seen that 10 quarters, all heavily infected with *Str. agalactiæ* and/or *Str. dysgalactiæ*, were treated, and that in 6 of these there were advanced clinical symptoms. In 3 of these latter and in the 4 quarters which were mildly affected the milk became completely normal, or at most showed a few tiny flocculi, within 1 week or less after treatment. The bacterial counts were also markedly reduced, many becoming negative for various periods, but in most cases the infection was not permanently sterilised. (The possibility of reinfection cannot be entirely dismissed, since the experiment was carried out primarily to test the tolerance of the udder for the products concerned.) It should be emphasised that in all cases, except one, only 1 injection was given.

TUBERCULOSIS EXPERIMENT

In Vitro Experiments.

A. *In Vitro.*

The *Ps. p.* products used were (a) crude dipoid, prepared by chloroform extracts of 6-weeks-old glycerin Bonillon cultures, after their acidification (as described earlier); (b) reconstituted freeze-dried 6-weeks-old glycerin broth cultures (killed at 60° C. for 1 hour).

(a) *Crude Lipoid.*

The dilution method seemed to be the most suitable for this experiment. A row of conical (Erlenmeyer) flasks of 300 ml. capacity was set up, one flask containing 90 c.c. of synthetic medium and the remainder the same medium, to which was added lipoid to give concentration of 1/100, 1/500, 1/1,000, 1/5,000, 1/10,000, 1/20,000, 1/50,000, 1/100,000, and 1/500,000 respectively.

These were inoculated with the human strain, D.T., 2 portions of 10-days-old pellicle from 7 per cent. glycerin bonillon being used as inoculum.

The results are shown in Table VIII and the photographs.

TABLE VIII.

M.tuberculosis, in vitro experiment.
Lipoid concentration in synthetic medium

Time	1/100	1/500	1/1,000	1/5,000	1/10,000	1/20,000	1/50,000	1/100,000	1/500,000	Control
7 days	—	—	—	—	—	∓	∓	+	+	+
14 days	—	—	—	∓	∓	∓	±	+	+	+
21 days	—	—	—	∓	∓	∓	±	+	++	++
5 weeks	—	—	—	∓	∓	∓	+	++	+++	+++
7 weeks	—	—	—	∓	∓	±	++	+±+	+++	+++
Transplant	—	—	—	slow	++	+++	+++	+++	+++	+++

A viability test was carried out at 7 weeks by transplanting pellicles and sediments (from the first 3 flasks) on to fresh medium. The cultures from the 1/100-1/1,000 flasks (inclusive) proved to be sterile, that from the 1/5,000 flask showed a markedly reduced and slow growth.

XUM

Comment. Crude lipoid appeared to be bactericidal against *Mycobacterium tuberculosis,* up to a dilution of 1/1,000, inclusive, in the medium used, and definitely bacterostatic up to 1/20,000 for a period of 7 weeks at 37° C., and partly bacterostatic up to 50,000 for 3 weeks.

(b) *Reconstituted freeze-dried killed culture.*

The reconstituted freeze-dried, heat killed culture was tested in the same way, the concentrations ranging from 1/5, 1/10, 1/25, 1/50, 1/100, 1/200 to 1/500.

Result. There was no growth after 7 weeks' incubation at 37° C. up to 1/50 dilution. All higher dilutions showed eugonic growth as in the control. The viability test proved that the sediment in 1/50 was still alive, and thus indicated the end point for bacteriocidal activity at 1/25 and for bacterostatic action at 1/50 for 7 weeks at 37° C.

In Vivo Experiment

Twenty guinea-pigs, in cages of two, were divided in 2 groups of 8 guinea-pigs each and one control group of 4.

Test Dose. 0.1 mg. of an equal mixture of *M. tuberculosis,* human type (strains P.N., D.T., and C.), suspended in 0.5 ml. normal saline, was injected intramuscularly in the right hind thigh.

Treatment. 50 mg. of crude lipoid in 5 ml. distilled water at a pH. of 7.8. Three injections at intervals of 1 week were originally intended, but this project could not be carried out, due to illness of the author. Half were treated by a single intramuscular injection at the same place as the infecting dose to allow direct action; half were treated in the other leg.

Result. The guinea-pigs were killed and autopsied after 6 weeks.

All controls showed advanced generalised tuberculosis. Of the guinea-pigs treated locally, one which was autopsied at 6 weeks and one which died at 4 weeks from a non-specific hæmorrhagic enteritis were free from tuberculosis bacterologically and histologically. The post-mortem findings in the remaining 6 guinea-pigs had to be classified as a mild general type of tuberculosis, but of much lower degree, with the lesions in the various organs less numerous, smaller and less active than in the controls or those treated in the opposite leg.

The lesions in those treated in the opposite leg appeared to fall about midway between those treated locally and the controls.

Discussion

It has long been known that *Ps. pyocyanea* and its metabolites possess antibiotic properties for certain other organisms, and that these are in the main due to pyocyanine, α-oxy-phenazine and a group of lipoidal substances. Pyocyanine, though strongly bactericidal, cannot be employed therapeutically on account of its high toxicity. α-oxy-phenazine, on the other hand, was already known to combine low toxicity with high bactericidal activity, whilst the lipoid substances were

also known to be bactericidal. Little was known, however, of the range of activity of these substances against the organisms of importance in animals, and nothing of their therapeutic value.

The production of α-oxy-phenazine presented no great difficulties. The general lines of preparation had already been laid down, and with some modifications of existing methods a crystalline product of high purity was prepared and shown to have an activity which embraced most of the organisms commonly pathogenic to animals. The yields, however, were not large, and it soon became obvious that when produced from cultures it would not compete with an antibiotic of the activity of penicillin against organisms susceptible to the latter. It was known, however, that it could be synthetically prepared, and *in vivo* tests were therefore pursued in *Br. abortus* infections and bovine streptococcal mastitis. The results in guinea-pigs infected with a dose of *Br. abortus* which infected all controls were encouraging; a little over half of the treated animals were cured, many by a single dose. This dose had, however, to be relatively large. The results of the subsequent experiments in cattle were somewhat disappointing. It should, however, be noted that in most cases only one treatment was given, and it was perhaps too much to hope that the heavily-infected animals could thus be cured. It was hoped at one time that sufficient synthetically produced α-oxy-phenazine would become available, but although a little was obtained for comparative tests *in vitro* and *in vivo* in guinea-pigs, sufficient was never available for cattle. The experiments in mastitis infection in cattle gave better results, but could not be pursued for the same reason. It is unsatisfying to leave the work at this point, because it is known that with many other agents, e.g., the sulphonamides and penicillin, a minimum concentration has to be kept up for a considerable period and continued treatment might have been effective. Nevertheless, it seems likely that such treatment would always be costly unless the synthetic production of α-oxy-phenazine becomes a commercial proposition.

The production of the lipoidal fractions presented more difficulty in the first place. The introduction of the method of obtaining it direct from liquid cultures after removal of the viscosity by acidification to pH. 3.5, with or without subsequent freeze-drying, made it possible, however, to obtain this fraction more conveniently than had hitherto been possible, whilst the high yield (300-400 mg. per litre of culture) of bactericidal material and its wide range of action gave promise that this product might have a future in chemotherapy. Much of the unwanted pyrogenic and toxic portions were also got rid of by the saturation with ammonium sulphate. Nevertheless, the lipoid obtained still had considerable toxicity, and purification by the way of its lead salt remains a necessary process if treatment is to be repeated or the material is to be left for long in the udder.

The fact that over 50 per cent. guinea-pigs infected with *Br. abortus* were cured by the lipoid was again encouraging, but attempts to reproduce these results in cattle with single doses were unsuccessful. Unfortunately, the various methods of preparation referred to above were not in operation at this time, and sufficient lipoid was not available for repeated treatment.

The *in vitro* and *in vivo* tests have indicated that the antibacterial activity of α-oxy-phenazine is relatively higher than that of the lipoid (at the degree of purification as obtained in the course of this study). Its diffusibility through solid medium is also better, whilst the required therapeutic tissue concentration is easier

XUM

obtained, because it is better tolerated. α-oxy-phenazine, whether locally or parentally applied, was less toxic and irritant than the crude lipoidal fraction, which, e.g., was tolerated in the cow's udder for only 1-2 hours without considerable local discomfort.

Serious reactions were also observed in a considerable proportion of guinea-pigs treated intramuscularly (but not intraperitoneally) with the largest dose, 50 mg., of crude lipoid. The same reaction, but usually of a milder nature, was also observed in some guinea-pigs treated with 25 mg., but not in those given 10 mg. The reaction appear to be a pruritus, which, in some cases, particularly with the largest dose, was so severe that it led to automutilation of the feet. It was not possible at this stage to prepare sufficient of the purified lipoid for use on guinea-pigs, and no intramuscular injections were therefore made with this product. The intravenous tolerance tests in mice suggest, however, that it is of much lower toxicity, and that it might be more safely injected intramuscularly in guinea-pigs. The point, however, should be made that this sequel in guinea-pigs was not seen after a single injection, but only after two or more injections at the same site. Repeated injection of α-oxy-phenazine at the same site with intervals of a few days also produced some irritation, though not of the same severity as with lipoid. In view of the fact that both α-oxy-phenazine and lipoid are tolerated in large doses by mice intravenously, and by guinea-pigs intraperitoneally, it seems possible that the local effects were due to the non-specific effect of the repeated injection of a large volume (50 mg. in 5 ml. and 25 mg. in 2.5 ml.) of alkaline solution. It should also be recalled that α-oxy-phenazine, though not the crude lipoid, could be left indefinitely in the cow's udder, a more sensitive organ than most. The high alkalinity referred to above cannot be avoided in the case of α-oxy-phenazine, since its critical point of precipitation is pH. 7.2-7.8, according to the concentration, nor entirely with lipoid, because solutions of this product have also to be kept just on the alkaline side of neutrality.

The activity of both products was unimpaired by the presence of blood in *in vitro* tests, whilst the results in *Br. abortus* infections in guinea-pigs suggest that they are not affected by tissue fluids. Moreover, α-oxy-phenazine (1 :500 in borate buffer) infused into the udder was demonstable colorimetrically for 3 hours and remained at the therapeutic concentration level for 2 hours, in spite of further dilution by milk secretion.

The curious sudden rise of *Br. abortus* agglutinins in the whey which was noticed after each infusion of both lipoid and α-oxy-phenazine is not easy to explain. Some direct agglutinature effect was at first considered, but none could be demonstrated with either product *in vitro*, and an actual increase in agglutinin content seems more likely. The facts that the peak of the whey agglutinins in the particular quarter never reached the level of the blood serum titre, and that this rise occurred in quarters free from specific bacteria and with a previously negative titre, seem to support the view that the blood milk barrier is loosened in some way which allows the passage of specific antibodies into the milk.

A comparative *in vitro* test between the crude lipoidal product and penicillin* showed that 1 mg. of lipoid was equivalent to 200 Oxford units of penicillin, that is to say, that weight for weight it had about 1/10 the potency of penicillin, taking the unitage of penicillin as 1,600-2,000 Oxford units per mg.

* The standard penicillin for this assay was kindly supplied by Mrs. Wilson.

XUM

Summary

(1) α-oxy-phenazine and a series of lipoidal products have been prepared from cultures of *Ps. pyocanea* on solid and in liquid media.

(2) *α-oxy-phenazine.*

The α-oxy-phenazine was obtained in a crystalline state in a high degree of purity and was shown to be active *in vitro* against the following organisms: *C. pyogenes, V. fœtus, P. bovis, B. anthracis, Str. agalactiæ, Str. dysgalactiæ, Staph. aureus, Br. abortus, B. coli, S. choleræ suis, Cl. Welchii, Cl. septique, Cl. chauvœi* and *Cl. tetani.* The bactericidal concentration varied according to the organism and time of contact from 1 :10,000 to 1 :1 million (within 5 hours), and from 1 :1,000 to 1 :20,000 (within 15 minutes). (Table No. I.)

Br. abortus infections in guinea-pigs. The intramuscular injection of α-oxy-phenazine, once or twice, 1-7 days after infection, prevented the development of *Br. abortus* infection in approximately half of a large number of guinea-pigs which had been given a test dose which infected all controls.

Br. abortus infections in cows. Single infusions of 500 ml. of α-oxy-phenazine at concentrations of 1 :500 to 1 :1,000 into udders excreting continuously large numbers of *Br. abortus,* accompanied in two cases by intravenous administration, was followed by short or longer intervals of absence of *Br. abortus,* but no permanent sterilisation followed.

Bovine mastitis. Udders infected with *Str. agalactiæ* or *Str. dysgalactiæ* were treated by introduction via the teat canal of 500 ml. of solutions estimated to contain 50-500 mgs. of α-oxy-phenazine. Clinical symptoms usually disappeared within two to four days, even in quarters which had previously given thickly clotted secretion or pus, the milk becoming normal. Two bacteriological cures were observed after a single infusion, but usually sterilisation was limited to periods lasting some days. The bacterial counts were considerably reduced, and in many cases the injections could only be detected by the enrichment method for a considerable period. No reaction to injection was observed.

(3) *Lipoidal Product.*

The lipoidal products were obtained in a crude form by chloroform extraction of dried bacterial bodies or of whole lipoid culture. The crude lipoidal product was purified by way of its ether soluble lead salt.

These lipoidal products proved to be active against *B. anthracis, Br. abortus, Str. agalactiæ, Str. dysgalactiæ, Staph. aureus, human and bovine,* and *M. tuberculosis,* at concentrations ranging from 1 :500 (within 15 minutes) to 1 :500,000 (within 5 hours). Tables No. II and No. VIII.

Br. abortus in guinea-pigs. The therapeutic effect was almost identical with the results obtained with α-oxy-phenazine.

Br. abortus in cows. Two heavily-infected quarters became negative for 17 days after a single infusion of lipoidal extract, but generally the post-infusion recordings were variably positive and negative, although the bacterial counts tended to decrease considerably. Intravenous injection was well tolerated, but was also unsuccessful.

Bovine mastitis. The results of a single intramammary infusion of the lipoidal product were similar to those of α-oxy-phenazine, but this product could

XUM

only be left safely in the udder for 1 hour at therapeutic concentrations. Table No. VII.

(4) The udder infusions with α-oxy-phenazine or with the lipoidal product were in all cases followed by a curious sudden rise of the whey agglutination titre, lasting many weeks.

(5) *Tuberculosis in guinea-pigs.* A single dose of 50 mg. of crude lipoid administered by the intramuscular route simultaneously with the injection of a mixture of three human strains which set up generalised tuberculosis in all controls, reduced the severity of the disease both qualitatively and quantitatively. Two treated guinea-pigs were entirely negative, both bacteriologically and histologically.

Acknowledgments

I wish to express my thanks to Professor T. Dalling, Director of the Veterinary Laboratory, Ministry of Agriculture and Fisheries, for his encouragement in his work, and also for his granting me the permission and facilities to work in the above Laboratory.

I am greatly indebted to Dr. A. W. Stableforth, head of the Bacteriology Department, for his constant encouragement, interest and guidance in devising the scheme of the work and help in the final drafting of the paper.

I would also like to express my gratitude to many British colleagues for their co-operation and helpfulness during my stay in Great Britain, especially those of the Veterinary Laboratory, Ministry of Agriculture and Fisheries, and of the Royal (Dick) Veterinary College, Edinburgh.

REFERENCES.

Berger, F. M. (1944) : *Nature,* 1944, Vol. 154, 459.
Berger, F. M. (1945) : *British Med. Journal,* 1945, *1,* 116.
Birch—Hirschfeld, L. (1934-35) : *Z. f. Hygiene,* 1934-35. (1935) : *Z. Hygiene Infekt. Kr.,* 116, 304.
Bouchard, C. (1889) : *Compt. rend.,* 108, 713-714.
Dietrich, A. (1901) : *Z. Hygiene Infekt. Kr.,* 37, 115.
Emmerich, R., and Loew, O. (1899) : *Zbl. Bact.,* 26, 237.
Emmerich, R., Loew, O., and Korschun, A. (1902) : *Ibid,* 31, 1.
Emmerich, R., and Saida, quoted by L. Birch—Hirschfeld· *Zeitsch. f. Hygiene,* 1934.
Fleming, A. (1929) : *B. Jl. Exp. Path.,* 10, 226.
Fortineau, L. : *Annales de l'Institut Pasteur,* 1910 *Compl. rend de l'Academie des Sciences* (1914), 158.
Fukuhara, Y. (1909) : *Jap. Arch. f. Hygiene,* 71, 387.
Grundel and Wagner (1930-31) : *Z. Immun. Forsch.,* 69.
Hettche, H. O. (1931-32) : *Arch. f. Hygiene* (1931-32), 107.
Hosoya, S. (1929) : *C. R. Soc. Biol.,* Paris, 99, 771.
Klimoff, J. (1901) : *Z. Hygiene. Infekt. Kr.,* 37, 120.
Lewkowitsch, J. : *Chemical Technology and Analysis,* 1921.
Ohkubo, S. (1910) : *Z. Immun. Forsch.,* 5, 428.
Pasteur, L., et Jourbert, J. (1877) : *Compt. rend. Sci. Acad. Sci. Paris,* 85 (101-105).
Raubitschek, H., and Russ, V. K. (1908) : *Zbl. Bakt.,* 48, 114.
Stableforth, A. W. (1939) : *J. Comp. Path. and Therap.,* 49, 251.
Schoental, R. (1941)· *British J. Exp. Path.,* 27, 137.
Stokes, J., Peck, R. L., and Woodward, J. R. : *Proc. Soc. Exp. Biol., N.Y.,* 51, 126.
Twitchell (1940) : "Chemical Constitution of Natural Fats," T. P. Hilditch.
Vaerst, K. (1902) : *Zbl. Bact. Paras.,* 31, 293-317.
Waksman, S. A. (1941) : *Bact. Rev.,* Vol. 5, No. 3.
Woodhead, M. M., and Wood, C. (1889) : *Compt. rend.,* 109, 985-988.
Wrede, F., and Strack, Er. Hoppe and Seyl : Z.140, 1 (1928) *Ibid.,* 177, (1929) *Ibid.,* 181, 58.

XUM

EFFECT OF FEEDING GREEN FOOD TO THE PREGNANT SOW ON THE INCIDENCE OF PIGLET ANÆMIA

By R. BRAUDE, Ph.D., and A. S. FOOT, M.Sc.,

National Institute for Research in Dairying, University of Reading.

NUTRITIONAL anæmia in suckling pigs is known to be very prevalent where litters are reared indoors. Dosing the piglets with solutions of various iron salts, or, indeed, administering reduced iron, has been found to be very effective in either preventing or curing the condition. Although the daily dosing of young pigs during the second week of their life can be carried out quite quickly, the practice does not appeal to most farmers. Painting the sow's udder with solutions containing iron and also the giving of soil to the litters have been used to prevent anæmia, but both these methods suffer from the disadvantage that they are not very hygienic and not always fully effective. In these circumstances the endowment of the young pig at birth with those factors necessary to carry him over the critical period up to three or four weeks of age would appear to be very desirable. Attempts to do this by feeding iron preparations to the pregnant sow have been described by Hart, Elvehjem and Steenbock,[1] Foot and Thompson[2] and others. In all these cases, however, this method was found to be ineffective.

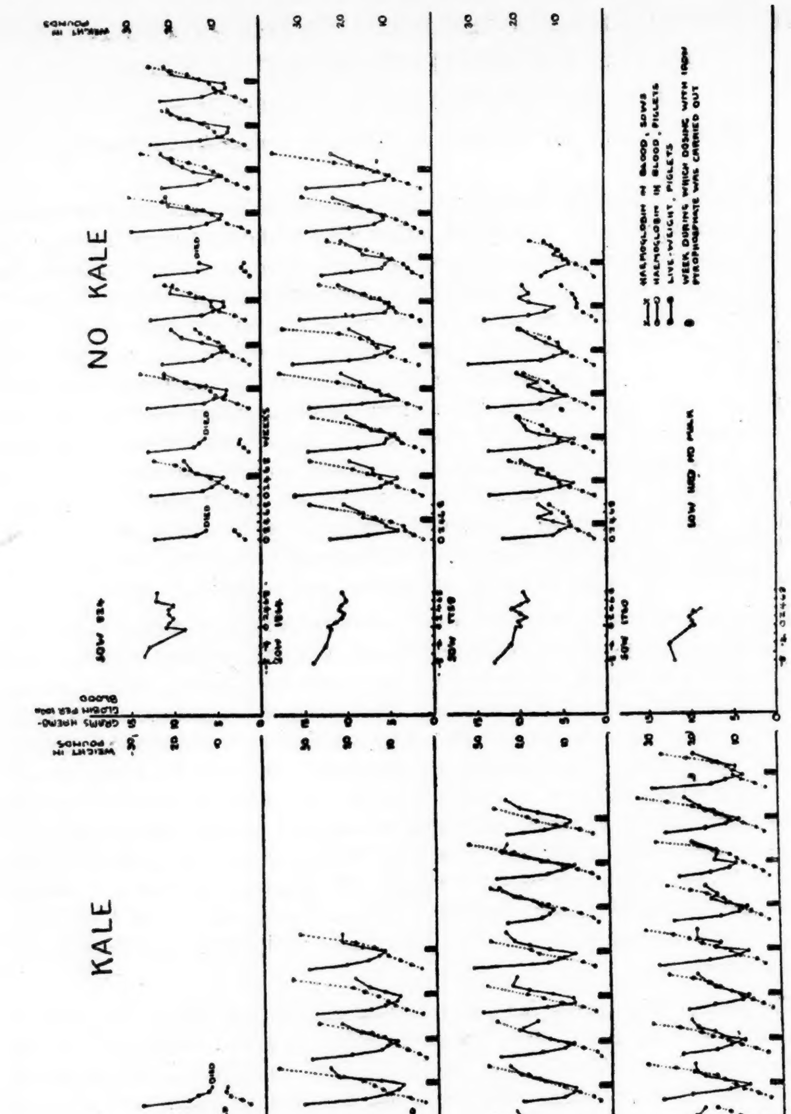
Recently, however, Venn[3] described experiments which appeared to show that the feeding of two plants of marrow-stem kale daily to pregnant sows resulted in litters free from anæmia. In view of the practical importance of this matter we have conducted experiments in which the hæmoglobin levels of litters from sows which received 7 lb. marrow-stem kale per day have been compared with those of litters from sows receiving the same diet, but with no kale. In the latter case a small additional allowance of meal was made to supply the major nutrients provided by the kale. All the sows received a meal mixture the composition of which was as follows: Wheatfeed 48, barley meal 20, ground oats 20, fish meal 10, minerals 2, all parts by weight. The mineral mixture was similar to that used by Venn,[3] namely, calcium carbonate 2, bone ash 2, salt 1, parts by weight. Each sow received also 5-10 g. of ferric oxide daily, according to her meal consumption.

The hæmoglobin curves for the individual pigs in four litters the dams of which received kale, and in three where no kale was given, are shown in the accompanying chart. The dam of the fourth litter receiving no kale farrowed normally, but failed to lactate after about one week, and the litter was removed from the experiment. There is no reason to believe that the lactational failure was due to the absence of kale.

The growth curves are also shown in the chart. In two pairs of litters the surviving piglets were given six doses of iron pyrophosphate containing 30 mg. Fe per pig per dose as a curative measure during the fifth week; the other three litters received the same treatment during the fourth week. Solid food for the piglets was introduced concurrently with the iron treatment.

The curves clearly show that in all cases the hæmoglobin level dropped to a dangerously low level, and the feeding of kale to the pregnant sow had no noticeable effect on the drop. The direct dosing of the piglets with iron pyrophosphate

XUM


NO KALE
KALE
HAEMOGLOBIN IN BLOOD, SOWS
HAEMOGLOBIN IN BLOOD, PIGLETS
LIVE-WEIGHT, PIGLETS
WEEK DURING WHICH DOSING WITH IRON PYROPHOSPHATE WAS CARRIED OUT
SOW HAD NO MILK
WEIGHT IN POUNDS
GRAMS HAEMOGLOBIN PER 100 ml. BLOOD
FARROWING
WEEKS
DIED

caused a very rapid recovery in hæmoglobin level, in some cases before the growth-curve had been significantly affected.

The hæmoglobin content of each sow's blood was estimated at six to eight weeks, and at four weeks before farrowing, at farrowing, and at weekly intervals during the suckling period (see chart). While there was a slight fall in hæmoglobin level in late gestation, probably a physiological anæmia of pregnancy, no sow reached a level that can be considered subnormal, and the hæmoglobin curves during suckling showed no consistent trend with either treatment.

1. Hart, E. B., Elvehjem, C. A., and Steenbock, H. (1930): *J. Nutr., 2,* 277.
2. Foot, A. S., and Thompson, S. Y. (1938): *J. Min. Agric., 45,* 452.
3. Venn, J. A. J. (1944): *Nature, 3889,* 591.

A SIMPLE TECHNIQUE FOR THE FIXATION AND STAINING OF *TRICHOMONAS* IN CULTURES AND VAGINAL MUCUS

By VERNON D. VAN SOMEREN, M.B.E., B.Sc., Ph.D.,
Zoologist, Central Veterinary Research Institute, Kabete, Kenya Colony.

Introduction

THE difficulty of obtaining perfect fixation and staining of *Trichomonas* in slide preparations has been emphasised by many writers from Dobell and O'Connor (1921) and Wenyon (1926) onwards.

It is possible, by the standard protozoological techniques of wet fixation and subsequent staining with an hæmatoxylin stain, occasionally to obtain good preparations of *Trichomonas* in culture media or body fluids; but it is very difficult by such methods to obtain smears in which all the protozoa are well shown. Usually a few only are sufficiently good for study purposes, and more often all the organisms are distorted and unrecognisable.

Trichomonas fœtus of bovines is no exception as regards the difficulty of fixation and staining of these trichomonad flagellates, as has been pointed out by Stableforth *et al.* (1937), Stewart (1938), and Bingham (1944). Numerous techniques have been tried on this species, of which the most successful appears to be the somewhat illogical method of Nöller (Witte, 1933), but these all suffer from the defects mentioned previously, only a few of the protozoa on each slide being suitable for study.

For several months now, strains of *Trichomonas fœtus* Riedmüller, 1928, obtained from bovines in Kenya, have been isolated and grown in pure culture in this laboratory by Dr. H. S. Purchase. Opportunity has been taken of this plentiful supply to find a technique suitable for making good slide preparations at will with the minimum of failures; and at the same time to discover a rapid fixation technique suitable for diagnosis from vaginal smears. Such a technique should obviate the necessity of examining fresh preparations for the living protozoa soon after collection of the sample, a procedure which is not always possible in the field.

XUM

Most of the standard protozoological methods were tried, either by wet fixation with known fixatives, such as Carnoy, Schaudinn, Bouin and their variant forms; or by vapour fixation with formalin and similar reagents.

These all failed to give satisfactory results with *Trichomonas fœtus* grown in S.E.B. or S.D.B. media (media quoted by Andrews and Lyford, 1940). The chief faults were overfixation, distortion during staining, and the fact that the outer surface of this species of *Trichomonas* appears to become very sticky when fixed, with a resultant accumulation of culture medium débris all round the organism on the slide.

Finally, simple wet fixation with iodine solution after the manner of Kent (quoted in Bolles-Lee, 1928) was tried, the film being subsequently dried and stained with Giemsa or hæmatoxylin, and by this method satisfactory preparations may be obtained as often as desired with a singular lack of difficulties or failures.

Technique

The iodine solution technique employed is as follows :—

(1) One drop of the culture medium containing living *Trichomonas* is placed in the centre of a well-cleaned, grease-free slide.

(2) One drop of freshly made Weigert's iodine solution (1 per cent. iodine in 2 per cent. potassium iodide) is then added to the culture drop and gently mixed.

The flagellates are killed instantaneously, and gradually take up the iodine, staining a golden brown with the nucleus and flagella sharply defined. They may be examined readily in this stage for gross morphology, but subsequent procedure for more permanent staining is carried out as follows :—

(3) The mixed drop is spread *very gently* over the slide with a needle in the form of a "thin-thick" smear, and allowed to dry completely. The drying may take place either at room temperature, or may be hastened by placing in an incubator at 37-40° C. The brown colour of the iodine gradually disappears as the slide dries.

(5) The slide is then taken down through lower alcohols to distilled water, in which it is rinsed for ½ minute.

(6) The smear is stained for 60 minutes in 1/25 Giemsa. This prolonged dilute stain gives much clearer results than staining for a shorter time in stronger Giemsa.

(7) The stained smear is differentiated by a ½-minute rinse in distilled water.

(8) The slide is dried and examined with a 1/12 oil immersion objective as for blood films; but as a preliminary to high-power study, the *Trichomonas* may be picked out readily by examining with a 2/3 objective. The best ones are found usually in the thinner portions of the smear.

Two points are of importance in this technique. Firstly, the iodine solution must be used as fresh as possible; solutions kept for a month or more, even in tightly stoppered bottles, produce very imperfect fixation. The solution used must possess strongly the characteristic odour of iodine.

Secondly, the mixing and spreading must be carried out as gently as possible.

XUM

Rough spreading breaks off the flagella, which appear to become very brittle after fixation by iodine.

The flagellates show the characteristic Romanowsky staining reactions by this technique. The cytoplasm is stained blue, the nucleus reddish, the anterior flagella, undulating membrane and axoneme red, the basal granules a dark purple. The cytostome is rarely visible in such fixed preparations and the basal fibre is not stained; the axostyle is, however, clearly visible in the blue cytoplasm as a colourless rod, often with reddish granules along its edge and a deeply staining red ring near the posterior tip. The technique is not very suitable for chromosome study; the nuclear fixation, while sharp, is similar to that produced by alcohol only, in that the nuclear material tends to be clumped together.

Slides prepared by iodine fixation still stain well after having been dried for 72 hours, or having remained in absolute alcohol for 12 hours, but they stain badly if they have been left in water (after alcohol treatment) for 12 hours. There is no difference in staining reaction between slides which have been dried slowly at air temperature and slides dried quickly in an incubator.

The technique works satisfactorily as often as desired with *Trichomonas* in S.E.B. medium, but I have been unable to obtain good preparations with this technique with cultures in S.D.B. medium. Smears prepared from S.D.B. cultures do not appear to dry properly after iodine treatment, and the *Trichomonas* are distorted; this is perhaps an effect of the increasing sugar concentration as the drop dries and the hygroscopic effect of the partially dry smear.

Instead of the second fixation of the dried smear in absolute alcohol, alcohols of 95 per cent. or 70 per cent., but not lower strength, may be used; but absolute alcohol gives a somewhat clearer result.

Staining with Heidenhain's iron-hæmatoxylin method, instead of Giemsa, gives very clear-cut morphological pictures, particularly if Shortt's modification of the hæmatoxylin stain is used (with carbolic acid, Bolles-Lee, 1928). The best results have been obtained by mordanting the smear (after alcohol treatment and subsequent rinsing in distilled water) for 24 hours in 2½ per cent. iron alum and then staining for 24 hours. The stained smear is then differentiated in ½ per cent. iron alum, rinsed in distilled water, washed in tap water, counterstained if required, taken up through the alcohols, and cleared in cedarwood oil. This last reagent is important, as clearing in xylol has invariably produced distortion of the protozoa. The smear is then mounted as usual in neutral balsam.

I have never experienced difficulty with the dried smear becoming detached in subsequent processes, even with thin S.E.B. culture fluid.

The various structures are sharply differentiated with Heidenhain stain, particularly the basal fibre, which stains intensely black. As is usual with any dried smear stained subsequently with iron-hæmatoxylin, the nucleus is not so densely stained as in preparations kept wet; but differentiation is sharp and usually a well-marked nucleolus can be distinguished, though with iodine fixation chromosome pictures are unsatisfactory. Basal granules are stained distinctly.

Staining with Delafield's hæmatoxylin after iodine fixation has also been found to produce good results. The staining is, however, very poor if the fixation of the dried smear is carried out in alcohol; the best results have been obtained by heat fixation of the dried smear, using the melting point of a small crystal

XUM

of urea placed on one end of the slide to indicate when a suitable temperature has been reached. The heat-fixed slide is then rinsed directly in distilled water and stained for 12 hours in 1/10 aqueous Delafield's hæmatoxylin acidulated with two drops of glacial acetic acid per 100 c.c. The stained slide is differentiated in 0.5 per cent. acid alcohol, dehydrated and cleared, again using cedarwood oil instead of xylol, and mounted in balsam. This technique shows all the structures in a similar manner to Heidenhain, including the basal fibre, which stains a deep purple.

Trichomonas in Vaginal Mucus

The iodine fixation technique works equally well for *Trichomonas fœtus* in bovine vaginal mucus, and the method is of use for diagnostic purposes when fresh smears cannot be examined immediately for living protozoa. It may be of value as well for examination of the human species of *Trichomonas*, but this has not yet been tried.

Owing to the tenacious nature of most bovine vaginal mucus, however, slight modifications of the technique have been found necessary to produce good results. The iodine solution must be very thoroughly, but gently, mixed with the mucus before spreading, and the smear should be made as thin as possible. It is then dried, refixed in absolute alcohol, and taken down to distilled water as before.

Subsequent staining with 1/25 Giemsa must then be prolonged for at least three hours, followed by a 10-second rinse in distilled water. With a staining period shorter than this, the flagella are not stained, probably because, on fixation, they become surrounded with a thin layer of fixed mucus through which the stain penetrates only slowly.

The final picture is as with *Trichomonas* in culture after iodine fixation and Giemsa staining, though often the edges of the body stain a deeper blue than the cytoplasm. Among the cell detritus found in vaginal mucus smears, the *Trichomonas* are picked out readily because of the characteristic blue cytoplasm; identification is usually rendered easy, even if the flagella are not well stained, by the axostyle, which appears as a colourless rod in the cytoplasm.

Notes on the Morphology of *Trichomonas* fœtus Riedmüller, 1928

The general morphology of *Trichomonas fœtus* of bovines has been well described by Riedmüller (1928), Witte (1933), and Stableforth *et al.* (1937), and the following notes give only a brief account of the morphology of the strain examined by means of this iodine fixation technique. As will be evident, this technique gives morphological pictures in all essential respects similar to those obtained by other workers using more complex methods.

The stained preparations show the diversity of shape associated with this and other species, as noted by Bishop (1931) and the above authors. Elongate forms, pear-shaped forms, "hour-glass" forms and almost spherical forms are common; these two latter types are found only in old cultures, as has been noted by previous workers.

The cytoplasm may be much vacuolated, especially in older forms, but the deep staining granules in the cytoplasm described by Stableforth *et al.* (*loc. cit.*) have not been seen in this strain. As with other fixation techniques, the cytoplasm is rarely visible in fixed preparations.

XUM

FIG. 1.—*Trichomonas fœtus*, iodine fixation, stained iron-hæmatoxylin. Ex. S.E.B. culture. x 1300.

FIG. 2.—*Trichomonas fœtus*, iodine fixation, stained iron-hæmatoxylin. Ex. S.E.B. culture. x 1300.

FIG. 3.—*Trichomonas fœtus*, iodine fixation, stained iron-hæmatoxylin. Ex. S.E.B. culture. x 1300.

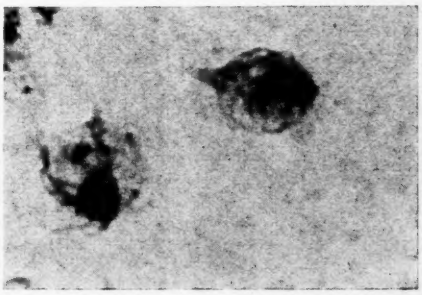


FIG. 4.—*Trichomonas fœtus*, iodine fixation, stained Giemsa. Ex. S.E.B. culture. x 1300.

XUM

XUM

TABLE 1.

Measurements of *Trichomonas fœtus* Riedmüller, 1928. Measurements in μ.

	van Someren, ex. S.E.B. culture, iodine-fixed.		Riedmüller, 1928.		Witte, 1933.		Stableforth, 1937.	
	Range	Average	Range	Average	Range	Average	Range	Average
Length (anterior end to posterior tip of axostyle)	12.0-17.5	14.5	10.0-25.0	—	15.0-22.0	—	8.0-18.0	Majority 12.0
Greatest breadth	6.0-11.0	8.7	5.0-10.0	—	3.0- 5.0	—	—	About 7.0
Length of posterior spike of axostyle	0.0- 2.0	1.0	—	—	—	—	—	—
Greatest breadth of axostyle ...	1.0- 2.0	1.3	—	—	—	—	—	—
Length of nucleus	3.0- 5.5	4.4	5.0- 7.0	—	—	—	—	—
Breadth of nucleus	2.0- 3.5	3.0	2.0- 3.0	—	—	—	—	—
Distance from posterior edge of nucleus to posterior tip of axostyle	6.5-10.5	8.4	—	—	—	—	—	—
Length of basal fibre	8.0-15.0	9.3	—	—	—	—	—	—
Length of free flagellum of undulating membrane	6.0-15.0	9.9	—	3 times body length when stretched.	—	—	—	—
Anterior flagella, average length and range of average length ...	9.0-16.3	10.9	—	15.0	—	16.0	—	Each one about as long as body.
Anterior flagella, range of length of individual flagella	7.0-18.0	—	—	—	—	—	—	—
Average difference in length between long and short flagella, and range of differences ...	0.0- 5.0	3.3	—	—	—	—	—	—

TABLE 2.

Anterior flagella measurements of 24 *Trichomonas fœtus*, iodine fixation. Measurements in μ.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
10	12	13	12	13	9	14	13	12	13	16	15	13	11	18	13	12	12	12	10	10	17	11	12
10	10	12	10	11	7	13	12	9	11	13	12	11	9	16	10	10	8	11	10	9	13	9	10
10	8	10	10	10	6	10	9	7	9	11	10	11	9	15	8	9	7	9	8	9	?	7	8

As noted by Riedmüller and Witte (*loc. cit.*), the anterior basal granule is multiple, consisting of 3-4 granules closely approximated. A parabasal body has not been observed. The axostyle originates just behind the basal granules and extends to the posterior tip of the body, the nucleus lying to one side of it at the anterior end, which is somewhat swollen. Small dark staining granules may occur along the edges of the axostyle, particularly in the posterior half.

All the above-mentioned authors note the characteristic appearance of the posterior tip of the axostyle in *T. fœtus*, though not all their diagrams show it well. This strain also shows this characteristic feature, which consists of a ring of deeply staining chromatin-like material surrounding the posterior tip of the axostyle in the cytoplasm. This material may occur in the form of a short band, on each side of the axostyle tip, which appears discontinuous. Posterior to this deep staining ring, the axostyle tip narrows suddenly into a well-marked spike-like prolongation, though this may be absent in some individuals.

Like all other strains of *T. fœtus* previously described, this strain is 3-flagellate only, and no variant individuals have been seen. Unlike other strains of *T. fœtus* described, however, an individual of this strain rarely has all three flagella of the same length; there is usually one long, one medium length, and one short one (*see table*). This is a feature hitherto recorded only in *T. prowazeki* (Bishop, 1931).

Binary dividing forms are not uncommon, but multiple division has not been observed in this strain.

A table is appended giving the average and range measurements of 25 *Trichomonas fœtus* picked at random on a slide made from S.E.B. culture, fixed with iodine and stained with Heidenhain's iron hæmatoxylin as described in this paper. As would be expected in a dried smear preparation, the breadth measurements slightly exceed those of previous authors.

Acknowledgments

I wish to thank Dr. H. S. Purchase for useful criticism and advice during the course of the work.

REFERENCES

Andrews, J., and Lyford, H. S. (1940): "Cultural Observations on *Trichomonas fœtus*," *Amer. J. Hyg.*, 31, 43-50.

Bingham, M. L. (1944): "Trichomoniasis—a Review of Recent Literature," *Vet. Bull.*, 14 (4), 113-146.

Bishop, Ann (1931): "The Morphology and Method of Division of *Trichomonas*," *Parasitol.*, 23 (2), 129-156.

Bolles Lee (1928): "Microtomist's Vade Mecum." 9th Edit., edited by J. Bronte Gatenby and E. V. Cowdry. *London: J. and A. Churchill.*

Dobell, C., and O'Connor, F. W. (1921): "The Intestinal Protozoa of Man." *London.*

Riedmüller, L. (1928): "Ueber die Morphologie, Uebertragungsversuche und klinische Bedeutung der beim sporadischen Abortus des Rindes vorkommenden Trichomonaden, *Zbl. Bakt. Abt. 1. Orig.*, 108, 103-118.

Robinson, E. M. (1937): "*Trichomonas* Infection in an Heifer, and a Brief Review of Abortion in Bovines due to *Trichomonas*," *J. S. Afr. Vet. Med. Ass.*, 8, 134-140.

Stableforth, A. W., Scorgie, N. J., and Gould, G. N. (1937)): "*Trichomonas* Disease of Cattle," *Vet. Record.*, 49 (8), 211-223.

Stewart, H. M. (1938): "The Staining of *Trichomonas fœtus* Riedmüller with Wright's Blood Stain," *J. Parasitol.*, 24, 473-474.

Wenyon, C. M. (1926): Protozoology, Vol. 1. *London: Baillière, Tindall and Cox.*

Witte, J. (1933): "Bakterienfreie Zuchtung von Trichomonaden aus dem Uterus des Rindes in einfachen Nährböden," *Zbl. Bakt. Abt. 1. Orig.*, 128, 188-195.

XUM

DO DOGS THINK?

By LORNA, COUNTESS HOWE, *Coopersale, Essex.*

My answer is "Yes," without shadow of doubt. I can quote many instances of this. Some people may think that I am drawing into "the realms of fancy." I am not, and I can bring many to support what I write. So many people think that instinct and preceding events are what people put down to thought. Well, they, I think, are totally wrong. I had once a very famous Labrador, afterwards Field Trial and Show Bench Champion, Banchory Bolo. I rescued him from an inglorious end, to make his name live for ever in the history of the Labrador. I am not going into how I did it, but I can assure readers that it was done by kindness and mutual love. When from the shy, timid creature he was (when I first made his acquaintance) he became sure of himself and of my love, he emerged a dog of great character. I spent endless hours with him teaching him not to suspect humans, as he did when I first met him. Finally, he became my shadow. Now take heed, those who doubt.

My husband and I were staying at a place in Wales, within motoring distance of our home. I had to go to London. It was when the rabies scare was on. I could not take Bolo, so I sneaked out, leaving him with my husband, to whom he was very attached. He left in our car, and Bolo jumped in, thinking, I suppose, that I should follow. When the car drove away he evidently thought he had been tricked. He sat on the floor of the car and would not speak or acknowledge my husband. On arrival at our home he jumped out and went straight to my room. Finding I was not there, he went to the kennels, and there he remained, visiting my room morning and evening, until my return. Then he met me with joyous welcome and then jumped up and welcomed my husband.

Surely that was well thought out.

Then, again, I had to go to London, and had to leave him behind. My husband wanted to take him out shooting, to amuse him. He got in the shooting brake with great joy, jumped out when it stopped, went round to smell everyone, found I was not there, and at once jumped back into the brake, and only growled when anyone attempted to get him out. He was driven back, a very disconsolate dog. Another instance of thought. I had a dog sent me with a very bad character. Taking the dogs for exercise, this dog made off to self hunt. I whistled and called, to no avail. Finally, the dog appeared (I had about twenty dogs out) and went in amongst the rest. He would not come to me. Bolo, of his own accord, went to him as a policeman might to a miscreant, and singled him out and literally marched him back to his kennel, and then sat down in the archway of the stable-yard. The bad dog came out again. I do not know what Bolo said, but it must have been something pretty potent, for the miscreant went into his kennel and Bolo returned to me and jumped up, as much as to say, "Well, that is finished." Once by sheer carelessness on my part I lost him. I had taken a taxi to the far end of Kensington Gardens, where he had never been before. I had a long letter, and was reading it when I missed Bolo. I did not know what to do. I went to the police station in Hyde Park, and finally, after an hour and more of distracted search, I went home to Grosvenor Crescent. The butler, who was devoted to Bolo, answered my ring, and said, "Are you looking for Bolo?" I said, "Yes." "Well," he said, "he came back over an hour ago, and I went down the area steps and let him in." My relief was enormous, but when Bolo met me he was exceed-

XUM

ingly cool and evidently thought it was through my carelessness he had been lost; and he was quite right! These are a few examples of thought. I could give many more about this great dog.

Now I will turn to a little griffon which I purchased nine years after Bolo's death. His mantle seems to have fallen on her small shoulders, for she is so like him in her ways. She is a "great lady" in the griffon world, weighing 6 lb. when I had her first, and is a full champion, but known to her friends and intimates as "Binkie the Beloved." Her sporting instincts attracted me. She killed three rabbits in the garden in the morning. A gun sends her wild with delight, and in her young days she would go out shooting and "sit" with three or four Labradors whilst the drive was on. Her attempts to retrieve a hare were partly successful, as she dragged it towards me and I went and met her, but I know she thought that she was entirely responsible for its retrieve. Can anyone wonder that I love this scrap of dogdom more than I care to say. Nothing daunts her.

One day a very great friend of mine said she would like to take her to show her to her mother. So Binkie went. I counted the hours and minutes until her return, thinking of the mutual joy of reunion. She came back. I was ill in bed, and instead of the joyous greeting I expected she just looked at me with her big eyes and then went and sat on my friend's knee, I am sure thinking, "You sent me away and I am angry with you."

Now, these things cannot be instinct; they must be reasoning. I could quote many more, but I have given examples of a great gun dog, and a great (in theory) toy dog. I have not written a word which cannot be substantiated by witnesses. People laughed at me for taking a toy dog to be my companion, but I have never regretted it. She is and always will be to me a sign of what a great heart in a small body can achieve. I dread the day of parting, but I hope, like all those whose animals are loved, that she and Bolo will give me joyous welcome when I reach the golden gate. That is, if *I* do. *They* should!

SOCIETY OF CHEMICAL INDUSTRY
AGRICULTURAL GROUP

MEETINGS for Season 1945-6 (all at 2.30 p.m.). Place of meetings to be announced later:—

March 19.—Insecticides: Chemical Constitution and Toxicity, by Dr. H. Martin.

April 23.—How Chemistry Helps the Stock-breeder.

May (date and time to be fixed).—Visit to the National Institute for Research in Dairying.

The meetings are open. All are welcome.

Erratum:

"Some Observations on the Incidence of Bovine Helminths in Plateau Province, Northern Nigeria," by J. F. A. Sprent.

In the above article (*Vet. J.*, 1946, *102*, 36-40), on p. 40, lines 2 and 3 should read:—

"Are lowest in the dry season, and in this season the average number of *Bunostomum phlebotonum* found in their carcases was about five times."

XUM